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Conditions Influencing Release of Granule Contents From Human Platelets in Citrated Plasma Induced by ADP or the Thrombin Receptor Activating Peptide SFLLRN: Direct Measurement of Percent Release of β-Thromboglobulin and Assessment by Flow Cytometry of P-Selectin Expression

Margaret L. Rand, Dennis W. Perry, Marian A. Packham, Cynthia H. Gemmell, Erik L. Yeo, and Raelene L. Kinlough-Rathbone

Department of Biochemistry (M.L.R., M.A.P.) and Department of Medicine (C.H.G., E.L.Y.), University of Toronto, Toronto, and Department of Pathology, McMaster University, Hamilton (D.W.P., R.L.K.-R.), Ontario, Canada

Contrary to a recent report [Rinder et al.: *Blood* 82:505, 1993], aspirin does inhibit the release of α-granule contents as well as inhibiting the release of dense granule contents by human platelets during ADP-induced aggregation in citrated platelet-rich plasma (PRP). Measurements were: percent release of ^{14}C-serotonin from prelabeled platelets, radioimmunoassay of β-thromboglobulin (βTG), and expression on the platelet surface of the α-granule constituent, P-selectin, by flow cytometry. During the second phase of ADP-induced aggregation, 69.0 ± 8.3% of βTG and 54.1 ± 4.6% of ^{14}C-serotonin were released (means ± SEM, n = 13); aspirin treatment reduced these values to 6.0 ± 1.2 and 1.0 ± 0.3%, respectively. In contrast, incubation of platelets with ADP without stirring caused only 6.7 ± 1.7% release of βTG and 2.1 ± 0.4% release of ^{14}C-serotonin; these low values were not appreciably affected by aspirin. During ADP-induced primary aggregation in PRP anticoagulated with FPRCH$_2$Cl (PPACK), only 4.7 ± 0.9% release of βTG and no detectable release of ^{14}C-serotonin occurred; aspirin had no effect. In both stirred and unstirred PRP, the thrombin receptor activating peptide, SFLLRN (50 μM), caused at least 75% release of the contents of both granules, which was partially inhibited by aspirin. Upon incubation of platelets with ADP (2–10 μM), the mean fluorescence intensity due to P-selectin was <14% of that induced by SFLLRN. In this unstirred system used for flow cytometry, aspirin treatment caused no significant inhibition of P-selectin expression. Thus, under conditions in which ADP does not cause secondary aggregation (physiological Ca^{2+} concentration or unstirred citrated PRP) release of the contents of both types of granules is less than 7% and aspirin is not inhibitory; the P-selectin expression associated with this low percent release is also unaffected by aspirin. However, aspirin *does* strongly inhibit the extensive release of both α-granule and dense granule contents during ADP-induced secondary aggregation in citrated PRP. © 1996 Wiley-Liss, Inc.

Key words: platelets, P-selectin, aspirin, granule release, ADP, SFLLRN

INTRODUCTION

Platelet aggregating agents have been designated as "weak" or "strong" on the basis of several criteria. Strong agonists such as thrombin can cause virtually complete discharge of the contents of both the α-granules and the dense granules, regardless of the concentration of Ca^{2+} in the suspending medium, and independent of stirring, aggregation, or the presence of an inhibitor such as aspirin that blocks the formation of the aggregating agent, thromboxane A_2 [2–5]. In contrast, the response of human platelets to a weak aggregating agent such as ADP is greatly influenced by these conditions. Upon rapid stirring in a

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Address reprint requests to Dr. M.L. Rand, Department of Biochemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

medium with a physiological concentration of Ca^{2+}, and in the presence of fibrinogen, ADP causes reversible primary aggregation with little release of granule contents and little or no formation of thromboxane A_2 [2]. However, in citrated platelet-rich plasma (PRP), or in an artificial medium with no added Ca^{2+}, primary ADP-induced aggregation is followed by secondary aggregation which involves the formation of thromboxane A_2 and the release of dense granule contents [2,6]. Secondary aggregation requires close platelet-to-platelet contact that takes place as aggregates form in a rapidly stirred system, and is attributed to the synergistic effects of thromboxane A_2 and released ADP [6]. Inhibitors of cyclo-oxygenase, such as aspirin, block ADP-induced secondary aggregation [2,6–8]. Thus, the title of a recent article [1] was surprising: "Aspirin does not inhibit adenosine diphosphate-induced platelet alpha granule release"; also unexpected was the conclusion that "aspirin treatment of platelets at doses that block dense granule secretion does not inhibit α-granule secretion to adenosine diphosphate" [1]. However, secretion of dense granule contents had been studied under conditions in which secondary ADP-induced aggregation would occur, whereas secretion of β-thromboglobulin (βTG, an α-granule constituent) had been studied in a non-stirred system. We therefore investigated the effect of aspirin on the release of βTG and ^{14}C-serotonin caused by stimulating platelets with ADP under aggregating and non-aggregating conditions in citrated platelet-rich plasma (PRP). For comparison we used a strong aggregating agent, the thrombin receptor activating peptide, SFLLRN [9], which releases platelet granule contents by a mechanism that appears to mimic that of thrombin [10,11].

Rinder et al. [1] also used the detection of P-selectin-positive platelets as an index of α-granule release. In unactivated platelets, P-selectin (CD62-P, GMP-140, PADGEM) is located on the inner aspect of the membrane of the α-granules; it appears on the platelet surface after platelet stimulation leads to fusion of the α-granules with the plasma membrane and discharge of their contents [12,13]. The introduction of flow cytometry to detect the presence of P-selectin on the surface of platelets stimulated with various agonists has led to an appreciation of the sensitivity of this method to detect a small percentage of platelets that have released some of their α-granule contents [14–16]. This type of analysis has also revealed such platelets in the circulation in conditions associated with an increased risk of thromboembolic events [17,18]. Several groups of investigators have shown that even low amounts of P-selectin on the platelet surface lead to increased platelet-leukocyte adhesion [19,20]. It is evident that detection of a slight release of platelet granule contents is the major interest of some investigators [1,21,22], whereas the percentage of the total granule contents that have been released is of interest to others. Confusion arises if detection of P-selectin is equated with release of α-granule contents, because the percentage of the contents released may be very low when P-selectin is readily detectable. We have attempted to resolve some of this confusion by assessing the appearance of P-selectin on the platelet surface upon treatment of platelets with ADP and SFLLRN.

MATERIALS AND METHODS

Materials

ADP, acetylsalicylic acid (aspirin), arachidonic acid (A 9673) and imipramine were from Sigma Chemical Co., St. Louis, MO. Paraformaldehyde (EM grade) was from Polysciences, Warrington, PA. The thrombin receptor activating peptide SFLLRN was synthesized by the Institute for Molecular Biotechnology (MOBIX) at McMaster University, Hamilton, Ontario, Canada. D-Phenylalanyl-L-prolyl-L-arginine chloromethylketone ($FRPCH_2Cl$, PPACK) and fluorescein isothiocyanate (FITC) isomer 1 on celite were from Calbiochem, La Jolla, CA. Pyridodisulfide R-phycoerythrin (R-PE) was from Molecular Probes (Eugene, OR). A2A9 (anti-GPIIb/IIIa monoclonal antibody [23], a generous gift of Dr. J. Bennett, University of Pennsylvania) was conjugated with FITC, and an irrelevant IgG monoclonal antibody (HL12-21, directed against a calcium-dependent epitope of factor IX) was labeled with R-PE, as described elsewhere [24]. KC4.1 (anti-P-selectin monoclonal antibody), an $IgG_1\kappa$ monoclonal antibody specific for P-selectin developed against thrombin-activated platelets as previously described [25], was labeled with R-PE as described elsewhere [24]. ^{14}C-Serotonin (5-hydroxy[side chain-2-^{14}C] tryptamine creatinine sulfate, 50 mCi/mmol) was from Amersham Canada, Oakville, Ontario. Radioimmunoassay kits for beta-thromboglobulin (βTG) (Code IM.88) were from the Kodak Clinical Diagnostics Division of Kodak Canada Inc., Toronto, Ontario.

Preparation of Platelets

Blood was obtained from donors who gave written, informed consent, and who had not taken medications affecting platelet functions for at least 2 weeks before donation. Citrated platelet-rich plasma (PRP) was prepared from blood in 0.38% sodium citrate (final concentration) by centrifugation at 200*g* for 15 min. Platelet counts were determined with a Coulter Counter (Model Z_F, Coulter Electronics, Hialeah, FL) and ranged from 310,000 to 500,000 per μL. Platelets were labeled by incubation of 30 mL of PRP with 0.5 μCi of ^{14}C-serotonin for 10 min at room temperature. For treatment with aspirin, PRP was incubated with 500 μM aspirin (final concentration) for at least 10 min at room temperature; samples that were not treated with aspirin were incubated with the same volume of isotonic saline. In one exper-

iment, blood was taken into $FPRCH_2Cl$ (final concentration 200 μM) as anticoagulant and PRP was prepared as described above. Imipramine (final concentration 5 μM) was added to all samples to prevent reuptake of ^{14}C-serotonin.

Aggregation and Measurement of Release of Granule Contents

One milliliter samples of PRP that had been prewarmed for 3 min at 37°C were aggregated by addition of ADP, SFLLRN, or sodium arachidonate. Isotonic saline was added to control samples. Changes in light transmission were recorded for 3–5 min with an aggregation module (Payton, Scarborough, Ontario, Canada), and then the samples were centrifuged for 1 min at 12,000*g* in an Eppendorf centrifuge (Brinkmann, Rexdale, Ontario, Canada). Supernatant samples were taken for measurement of ^{14}C-serotonin and βTG release. Percentage release was measured after subtraction of the amounts in the supernatant of unstimulated, control samples. For βTG, the percentage of the total platelet βTG that was in the supernatant platelet-poor plasma averaged 5.6 ± 1.4% (mean ± SEM, n = 8). To determine the total amount of βTG in PRP, samples were mixed with benzamidine (final concentration 100 μM), subjected to a freeze-thaw cycle and sonicated (3 pulses, 10 sec each) before dilution for radioimmunoassay.

Preparation of Samples for Measurement of Release and Expression of P-Selectin

The method of Rinder et al. [1] was followed. Each sample of citrated PRP was warmed to 37°C for 3 min, an aggregating agent (or its diluent) was added, the mixture was vortexed for 1 sec and then incubated at 37°C for 5 min. A 20 μL portion of each sample was taken for flow cytometry and fixed by the addition of an equal volume of 2% paraformaldehyde in phosphate buffered saline. The remainder of each sample was centrifuged and the supernatant was prepared for βTG assay as described above; total platelet content of βTG was also determined.

Flow Cytometry

Ten microliters of fixed platelet suspension was added to 50 μL of Hepes-Tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 16 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM Hepes, 1 g/L glucose, 2 g/L bovine albumin, pH 7.4) containing saturating concentrations of FITC-A2A9 and PE-KC4.1. Platelets were identified as being positive for FITC-A2A9 and within the single intact platelet window defined by forward and side light scatter characteristics. The percentage of platelets expressing P-selectin above that of background (irrelevant antibody PE-HL12-21) and the mean fluorescence intensity (in arbitrary fluorescence units) of the total platelet population were recorded. Antibody positive events were defined as platelets with a fluorescence intensity >95% of the signal associated with the irrelevant antibody, PE-HL12-21. Samples were analyzed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA) formatted for two color analysis using Becton Dickinson (San Jose, CA) LYSIS II software.

RESULTS

Table IA shows a strong inhibitory effect of aspirin on the percentage release of both βTG and ^{14}C-serotonin when platelets were aggregated in citrated PRP by concentrations of ADP that caused the second phase of aggregation to occur. Among the 8 donors used, there was considerable variation in the concentration of ADP required to cause the second phase of ADP-induced aggregation. One donor's platelets responded in this way to 1 μM ADP, whereas 10 μM ADP was required with platelets from another donor. Results shown in Table IA are for samples in which secondary aggregation occurred without aspirin, but was prevented by aspirin. Without aspirin, the percentage release of βTG, 69.0 ± 8.3%, was somewhat higher than the percentage release of ^{14}C-serotonin, 54.1 ± 4.6%, but aspirin had a strong inhibitory effect on the release of both these dense granule and α-granule constituents. The total amount of βTG averaged 69.2 ± 2.9 $\mu g/10^9$ platelets (mean ± SEM, n = 8).

To ensure that aspirin, under the condition used, blocked the formation of thromboxane A_2, platelets were stimulated with sodium arachidonate. Table IA shows that aspirin almost completely blocked the release of βTG and ^{14}C-serotonin induced by sodium arachidonate. Aggregation induced by sodium arachidonate was also completely inhibited by aspirin (results not shown).

In contrast to the results obtained with samples in the aggregometer, when platelets in citrated PRP were vortexed with ADP for 1 sec and then incubated for 5 min with no further stirring, release of both βTG and ^{14}C-serotonin was very low (6.7 ± 1.7% and 2.2 ± 0.4%, respectively) and scarcely affected by aspirin (Table IB). Measurements of P-selectin expression under identical conditions (see below and Table II) showed that in the vortexed and incubated samples, ADP-induced P-selectin expression was low and unaffected by aspirin.

We also measured ADP-induced release of βTG and ^{14}C-serotonin in PRP from blood anticoagulated with the thrombin inhibitor, $FPRCH_2Cl$; in this PRP, the concentration of Ca^{2+} is physiological, and even high concentrations of ADP induced only the primary phase of aggregation. As predicted from previous results [2], in this PRP, ADP-induced release of both βTG and ^{14}C-serotonin was very low without aspirin and inhibition by aspirin was insignificant (Table IC).

Table IA and B also shows results with the thrombin receptor activating peptide, SFLLRN [9]. This peptide was used instead of thrombin to avoid both the confound-

TABLE I. Effect of Aspirin on Release of βTG and ^{14}C-Serotonin During: A. Second Phase Aggregation by ADP and Aggregation by SFLLRN or Sodium Arachidonate in Citrated PRP; B. Incubation of Citrated PRP With ADP or SFLLRN; C. Aggregation of FPRCH$_2$Cl-PRP by ADP or Sodium Arachidonate*

		Release (% of total in platelets)			
		βTG		^{14}C-Serotonin	
Aggregating agent	n	No aspirin	With aspirin	No aspirin	With aspirin
A. Aggregation in citrated PRP (in aggregometer)					
ADP (1–10 μM)	13[a]	69.0 ± 8.3	6.0 ± 1.2	54.1 ± 4.6	1.0 ± 0.3
SFLLRN (50 μM)	5	92.6 ± 4.5	70.1 ± 7.0	81.5 ± 2.5	66.0 ± 1.6
Sodium arachidonate (500 μM)	3	91.4 ± 8.6	1.5 ± 0.4	57.3 ± 6.8	1.6 ± 0.2
B. Stimulation of citrated PRP (vortex 1 sec, 5 min without stirring)					
ADP (2–10 μM)	8[b]	6.7 ± 1.7	8.9 ± 1.8	2.1 ± 0.4	1.8 ± 0.3
SFLLRN (50 μM)	5	85.1 ± 8.1	77.6 ± 8.6	75.6 ± 2.1	69.2 ± 1.3
C. Aggregation of FPRCH$_2$Cl-PRP (in aggregometer)					
ADP (1–5 μM)	3[c]	4.7 ± 0.9	4.6 ± 0.7	0 ± 0	0 ± 0
Sodium arachidonate (500 μM)	1	77.5	1.5	56.0	0

*Total amount of βTG averaged 69.2 ± 2.9 μg/10^9 platelets (n = 8).
[a]Mean ± SEM of 13 samples in 8 experiments.
[b]Mean ± SEM of 8 samples in 5 experiments.
[c]Mean ± SEM of 3 samples in 1 experiment.

TABLE II. Effect of Aspirin on Expression of P-Selectin on the Platelet Surface Following Stimulation With ADP or SFLLRN for 5 Min*

Agonist (μM)	Percentage of P-selectin-positive platelets		Mean fluorescence intensity of total platelet population (arbitrary fluorescence units)	
	No aspirin	With aspirin	No aspirin	With aspirin
None	5.6 ± 1.5	5.8 ± 1.5	0.8 ± 0.3	0.8 ± 0.5
ADP, 2	12.9 ± 3.1	13.1 ± 3.2	2.6 ± 0.7	2.5 ± 0.8
ADP, 5	22.4 ± 5.7	22.6 ± 4.8	5.1 ± 1.4	5.1 ± 1.3
ADP, 10	30.1 ± 6.8	27.9 ± 6.0	8.0 ± 2.5	7.2 ± 1.7
SFLLRN, 50	86.9 ± 2.5	85.0 ± 3.5	52.6 ± 5.9	47.4 ± 6.4

*Mean values ± SEM, n = 6.

ing effects of fibrin formation which thrombin causes in plasma, and the inhibitory effects of the antithrombins in plasma. SFLLRN has been reported to stimulate platelets in the same way as thrombin [10,11]. Aspirin does not block thrombin-induced platelet aggregation and has little effect on the extent of release of platelet granule contents, although some inhibition can be demonstrated at low concentrations of thrombin [2–5]. Aspirin had a partial inhibitory effect on release when platelets were aggregated by 50 μM SFLLRN ($P < 0.005$ for βTG and $P < 0.0025$ for ^{14}C-serotonin, paired difference analysis, Table IA), but was not significantly inhibitory of βTG release when they were incubated with SFLLRN without stirring (however, $P < 0.0125$ for ^{14}C-serotonin release, Table IB).

Table II shows the results of 6 flow cytometry experiments assessing the expression of P-selectin on the surface of platelets stimulated with ADP, using the method of 1 sec vortexing followed by 5 min incubation at 37°C. Also shown are results with platelets mixed and incubated with SFLLRN in the same way. Only a few microaggregates form under these conditions.

The increase in the percentage of P-selectin-positive platelets induced by ADP was similar in the presence and absence of aspirin (30% with 10 μM ADP), and much lower than that observed with the strong release-inducing stimulus, 50 μM SFLLRN (Table II). With SFLLRN, the percentage of P-selectin-positive platelets was greater than 85% in both the presence and absence of aspirin.

The extent of expression of P-selectin, indicated by the mean fluorescence intensity of the total platelet population, was very low with ADP-stimulated platelets (2.6–8.0 for 2–10 μM ADP in the absence and presence of aspirin) compared with the mean fluorescence intensity of platelets stimulated with 50 μM SFLLRN (52.6 ± 5.9

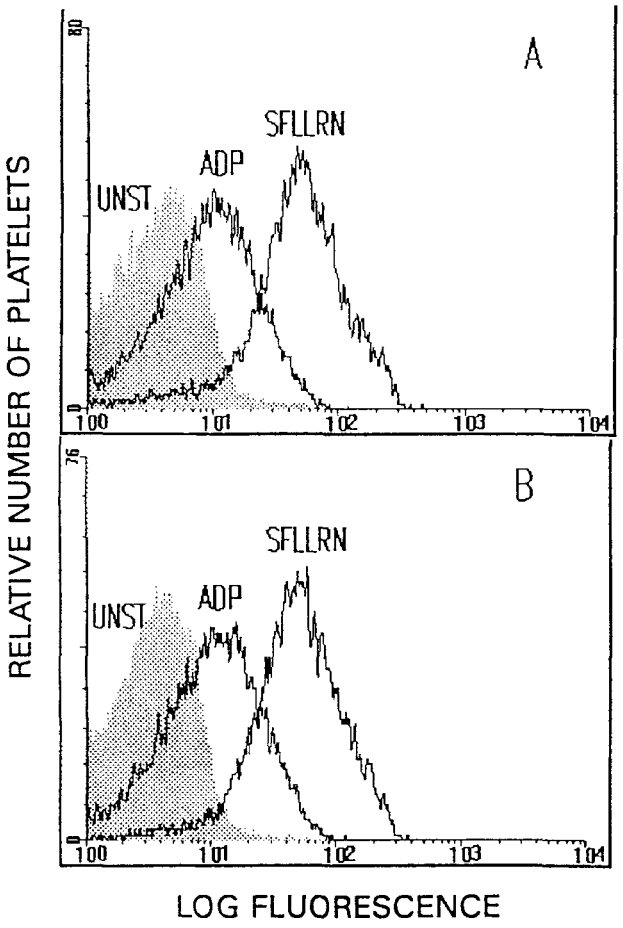

Fig. 1. Log fluorescence histograms showing P-selectin expression on the surface of (A) control platelets and (B) aspirin-treated platelets. Profiles are shown for unstimulated platelets (UNST), platelets stimulated with 10 μM ADP, and platelets stimulated with 50 μM SFLLRN. Representative of 6 experiments with similar results.

without aspirin and 47.4 ± 6.4 with aspirin) (Table II, Fig. 1). Thus, the extent of P-selectin expression induced by ADP was <14% of that induced by SFLLRN. This value agrees closely with the low percentage release of βTG that we observed with ADP under non-aggregating conditions; release of βTG caused by ADP was only about 8% of the release caused by SFLLRN (Table IB).

DISCUSSION

These results show that in rapidly stirred, citrated PRP (a medium in which many investigators test platelet aggregation), aspirin prevents ADP-induced secondary aggregation and has a strong inhibitory effect on the release of both an α-granule constituent, βTG, and a dense granule constituent, serotonin. Although there are many reports in the literature showing that aspirin prevents secondary ADP-induced aggregation and serotonin release in citrated PRP [2,6–8], very few investigators have measured the effect of aspirin on the release of α-granule constituents under these conditions. Our observations are in accord with the findings of Kaplan et al. [26] who showed in 1979 that aspirin ingestion by platelet donors completely inhibited ADP-induced release of platelet factor 4, βTG, and fibrinogen, all of which are constituents of the α-granules. Thus, under conditions in which ADP induces extensive release of granule contents and thromboxane A_2 formation, aspirin strongly inhibits the release of the contents of both α-granules and dense granules.

We also examined the effect of aspirin in two conditions under which ADP does not cause extensive release of the contents of either type of granule. These conditions were a brief mixing of citrated PRP with ADP followed by incubation at 37°C for 5 min (the technique used by Rinder et al. [1] for flow cytometric detection of P-selectin and for measurement of secretion of βTG), and ADP-induced aggregation in PRP from blood anticoagulated with $FPRCH_2Cl$ to maintain the physiological concentration of Ca^{2+} [2]. Under both these conditions, the release of both βTG and ^{14}C-serotonin was less than 7% of the total and the presence of aspirin had scarcely any effect on the values obtained. George et al. [27] have also shown little percent release of βTG at concentrations of ADP up to 50 μM in an unstirred system. When we compared our results with those of Rinder et al. [1], we noted that they did not provide a value for the total βTG content of the platelets. It seems probable that the percent release of βTG in their diluent treated samples may have been similar to the percent release of 5–10% that we and George et al. [27] observed; if so, our results do not disagree with those of Rinder et al. [1]. However, it was misleading to compare the lack of effect of aspirin on this low extent of release of βTG with the inhibitory effect of aspirin on the large percentage release of serotonin that occurred when platelets underwent ADP-induced secondary aggregation in rapidly stirred citrated PRP. The conclusion that "aspirin treatment of platelets at doses that block dense granule secretion does not inhibit α-granule secretion to ADP" [1] is based on an unjustifiable comparison; values for serotonin release were not obtained under the conditions in which βTG release was measured.

A report of Janes et al. [21] of partial degranulation of platelets by ADP in the absence of aggregation (brief mixing followed by incubation at 22–26°C) also does not provide a value for total βTG, merely showing that the values for released βTG (in $\mu g.l^{-1}$) parallel the values for % P-selectin-positive platelets as the concentration of ADP is raised. However, assuming a platelet count in PRP of 300,000/μL, and our value of βTG of 69 $\mu g/10^9$ platelets, the maximum percentage release of βTG they report for 10 μM ADP is about 10% of the total βTG. (It should be pointed out that values in the literature for total βTG vary somewhat, from 8.1 to 60 $\mu g/10^9$ platelets

[26,28–33].) Thus, these investigators also are showing the low release of βTG that occurs upon stimulation of platelets with ADP in an unstirred system. In a more recent report from the same group [22] a similar release of βTG caused by ADP in an unstirred system is shown, but the amount reported to be released by maximum stimulation with thrombin is much lower than we observed upon stimulation with SFLLRN.

Our results do indicate that release of the contents of the α-granules occurs to a slightly greater extent than release of the contents of dense granules in response to either ADP or SFLLRN. The significant inhibition by aspirin of release of granule contents induced by SFLLRN during aggregation in citrated PRP (Table IA) is likely due to inhibition of the thromboxane formation that would be induced by close platelet-to-platelet contact in this low Ca^{2+} medium; we have previously reported this effect when thrombin was used as the agonist [2].

It is evident that ADP-induced P-selectin expression can appear to involve a large percentage of the platelets when the percent release of α-granule contents, indicated by βTG release, is of the order of only 5–9%. It seems likely, therefore, that the P-selectin positive platelets have released only a small portion of their α-granule contents, and this conclusion is supported by the low mean fluorescence intensity of the platelets stimulated with ADP, which was less than 14% of the value for those stimulated with SFLLRN.

In agreement with the results reported by Rinder et al. [1], we found that the increase in the percentage of P-selectin-positive platelets upon brief mixing with ADP followed by incubation, was similar in the presence and absence of aspirin. Aspirin also had little effect on the mean fluorescence of the total platelet population. The values for expression of P-selectin on the platelet surface that were obtained with ADP are very low in comparison with the values obtained with the thrombin receptor activating peptide, SFLLRN. Shattil et al. [14] have also shown that 10 μM ADP causes much less expression of P-selectin on the platelet surface than does phorbol myristate acetate, which causes extensive release of granule contents. Comparisons of mean fluorescence intensity should be included with strong release-inducing stimuli that do not require stirring to cause the release reaction and result in maximal expression of P-selectin. In addition, the percentage release of either α-granule contents or dense granule contents induced by a weak agonist such as ADP in a rapidly stirred system, cannot be assumed to be the same as under the non-aggregating conditions in which P-selectin is measured by flow cytometry.

It is evident from our results with 50 μM SFLLRN and the results of Rinder et al. [1] with 0.01 U/mL of bovine thrombin, that aspirin has little effect on the thrombin-induced expression of large amounts of P-selectin on the platelet surface. This is not surprising because thrombin can act on platelets through signaling pathways that are independent of thromboxane A_2 [2–5]. Thrombin or SFLLRN also causes a large percentage of granule contents to be released in an unstirred system in which little or no aggregation occurs.

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